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Heterochromatin Silencing and Locus Repositioning Linked to Regulation of Virulence Genes in *Plasmodium falciparum*

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Summary

The malaria parasite *Plasmodium falciparum* undergoes antigenic variation to evade host immune responses through switching expression of variant surface proteins encoded by the *var* gene family. We demonstrate that both a subtelomeric transgene and *var* genes are subject to reversible gene silencing. *Var* gene silencing involves the SIR complex as gene disruption of *PfSIR2* results in activation of this gene family. We also demonstrate that perinuclear gene activation involves chromatin alterations and repositioning into a location that may be permissive for transcription. Together, this implies that locus repositioning and heterochromatic silencing play important roles in the epigenetic regulation of virulence genes in *P. falciparum*.

Introduction

In *Plasmodium falciparum*, the causative agent of the most severe form of malaria, gene families encoding proteins involved in immune evasion and virulence can be located in the subtelomeric regions of the 14 chromosomes (Gardner et al., 2002). The overall structure of *P. falciparum* chromosome ends is conserved with blocks of repeated DNA defined as telomere-associated repeat elements (TAREs) 1 to 6 found internal to the telomere (Figueiredo et al., 2000; Gardner et al., 2002). Subtelomeric *var* genes represent the most telomere-proximal genes and are located downstream of TARE6 (Rep20; Åslund et al., 1985; Gardner et al., 2002). These sequences are neighbored by *rifin* and *stevor* genes as well as additional *var* genes. A recent study has shown that *P. falciparum* chromosome ends cluster at the nuclear periphery (Freitas-Junior et al., 2000), and Rep20 (TARE6) has been implicated in this process (O'Donnell et al., 2002). The conserved structure of *P. falciparum* chromosome ends and their physical association may facilitate generation of diversity in virulence genes by promoting ectopic recombination.

Owing to their central role in immune evasion and pathogenesis *P. falciparum var* genes comprise the most important and best studied subtelomeric gene family (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). The *var* gene family encodes *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is expressed at the surface of infected erythrocytes and mediates binding to receptors on host cells. To avoid clearance by anti-PfEMP1 antibodies, *P. falciparum* can change the variant form of PfEMP1 expressed on the erythrocyte. This process of antigenic variation underpins the chronicity and virulence of this parasite.

There is only one *var* gene from which full-length transcript is derived in any one parasite, while other members of this family are essentially silenced (Scherf et al., 1998). Antigenic variation of PfEMP1 is generated through in situ activation of a silenced *var* gene and does not involve recombinational activities such as gene conversion into an active expression site. This mutually exclusive transcription of *var* genes and the ability of the parasite to switch expression to a different member is the primary basis of antigenic variation in *P. falciparum*. Most *var* genes are located in the subtelomeric region of each chromosome; however, there are also some in more central regions and there is evidence suggesting regulation of each subgroup may differ as each have distinct promoter regions (*upsA*, *B*, and *C*) (Gardner et al., 2002; Voss et al., 2003). To date, the molecular mechanisms underlying silencing and antigenic variation of *var* genes in *P. falciparum* are not understood.

In higher eukaryotes, chromosomal domains exist as euchromatin or heterochromatin due to regional differences in structure. Variations in DNA packaging influence the transcriptional activity of genes. Heterochromatin-like structures are also present at silenced yeast loci including telomeres and the subtelomeric silent mating type loci *HML* and *HMR* (reviewed in Lustig, 1998). The yeast SIR2 protein (silent information regulator 2) plays a central role in silencing by marking chromatin through deacetylation of histone tails (reviewed in Gasser and Cockell, 2001). The *P. falciparum* genome encodes a SIR2 homolog (PfSir2), although no functions have been assigned to this protein. In addition, no studies have determined the role of epigenetic mechanisms such as histone deacetylation and chromatin compaction in regulation and silencing of *var* gene transcription in *P. falciparum*.

To address perinuclear regulation of subtelomeric virulence genes in *P. falciparum*, we inserted the human dihydrofolate reductase (*hDHFR*) gene into the TARE6 region of chromosome 3. We demonstrate that expres-

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sion of this marker gene, as well as endogenous *var* genes, are epigenetically controlled and that locus repositioning and chromatin structure play a role in silencing and activation of these genes in *P. falciparum*.

Results

Integration of *hDHFR* into Subtelomeric Rep20 on Chromosome 3

To insert a marker of transcription into the subtelomere of a chromosome, we transfected *P. falciparum* parasites (3D7) with plasmid pHdhfr containing *hDHFR*-encoding resistance to the antifolate drug WR99210 (WR; Fidock and Wellems, 1997), under control of the calmodulin promoter (Crabb and Cowman, 1996). The plasmid pHdhfr included 506 bp of Rep20 for homologous integration into the subtelomere of chromosome 3 and the chloramphenicol acetyltransferase (CAT) sequence as a target for fluorescence in situ hybridization (FISH; Figure 1A). Transfected parasites were selected on WR to obtain 3D7/H with integration of pHdhfr next to a *var* gene. The structure of integration was confirmed by pulsed-field gel electrophoresis (PFGE; data not shown) and Southern blotting (Figure 1B) and revealed insertion of two plasmid copies 3.3 kb upstream of *var* gene PFC0005w on chromosome 3 (Figure 1). As control, we targeted the nonessential *Pfrh2a* gene (on chromosome 13) by transfection of *P. falciparum* (3D7) with pHdhfrΔ2a to generate a parasite line with two plasmid copies inserted (3D7Δ2a; data not shown) (Duraisingh et al., 2003).

To determine if the *hDHFR* gene maintained transcriptional activity without drug selection, the transfected parasite lines were cloned and grown for 50 generations in the absence of WR to obtain the lines 3D7/Hc1 and 3D7Δ2ac (Figure 1C). Parasite clones grown without drug selection had an IC_{50} for WR of 2 nM, while for those maintained on drug it was 30 nM (Figure 2A). Additionally, the shape of the curve was flattened for parasites grown without drug selection, suggesting a mixed population. Selection of these parasites on WR restored the typical curve, whereas no change was evident for control parasites (data not shown). We considered it likely that approximately 40% of the population had the *hDHFR* gene silenced or inactivated and that its subtelomeric location may be important for this variegated transcriptional state.

The Subtelomeric *hDHFR* Transgene Is Reversibly Silenced

To determine if a subpopulation of parasites contained a silenced *hDHFR*, we cloned 10 parasites (3D7/Hc1.1–10) and analyzed their sensitivity to WR (Figure 1C). Three lines were sensitive to 10 nM WR while seven were fully resistant, confirming the presence of two subpopulations. Two resistant and two sensitive clones were used for further analysis and each was grown on WR to derive the lines 3D7/Hc1.1$^+$, 3D7/Hc1.2$^+$, 3D7/Hc1.3$^+$, and 3D7/Hc1.4$^+$ (Figure 1C).

hDHFR was transcribed in the WR-resistant lines as shown by real-time RT-PCR but not in the sensitive parasites (Figure 2B). After selection on WR, however, the parasite lines showed high levels of *hDHFR* transcripts. These results suggest *hDHFR* in the absence of drug selection was silenced and that parasites with activated forms could be selected by growth on WR. Transcription of the adjacent *var* gene PFC0005w in RT-PCR was not affected by the transcriptional state of the neighboring transgene (Figure 2B).

One explanation for the silencing and activation of the inserted *hDHFR* was the locus or surrounding region had altered in a way that affected transcription or that copy number of the inserted plasmid was unstable. Telomere length has been shown to affect the activation of subtelomeric genes in mammalian cells (Baur et al., 2001). However, telomere length in the transfected lines with either active or inactive *hDHFR* genes was not different for all parasite lines (Figure 2C). Similarly, Southern blotting experiments showed that the DNA surrounding the plasmid insertion was not different and that the copy number of the plasmid was stable for all parasites irrespective of the transcriptional state of the *hDHFR* transgene (Figure 2C). These results suggest transcription of *hDHFR* inserted in the subtelomere of chromosome 3 is under epigenetic regulation. Additionally, the transcriptional state of this gene could be maintained through many mitotic divisions, an important feature of silenced chromatin domains and epigenetic inheritance (Grewal, 2000).

Silencing and Activation Is Associated with Alterations in Local Chromatin Structure and PfSir2 Function

The subtelomeric regions in other organisms have been implicated in recruitment of chromatin-modifying enzymes and assembly of repressed chromatin leading to silencing of nearby genes (Henikoff, 2000; Hsieh and Fire, 2000). For example, subtelomeric DNA of *S. cerevisiae* is organized into nucleosomes but is less accessible and more compact compared to bulk chromatin (Gottschling, 1992). Alterations in chromatin structure would alter accessibility to regulatory proteins and therefore the transcriptional state of a gene located in such regions. As in yeast, it has been shown that the *P. falciparum* subtelomeric regions display a regular nucleosomal organization (Figueiredo et al., 2000). We digested native chromatin with micrococcal nuclease (MNAse) in cells made permeable with NP-40 to determine differences in chromatin structure at the subtelomeric *hDHFR* locus in the active (3D7/Hc1.3$^+$) versus the silenced (3D7/Hc1.3) state (Figure 3A). The digestion patterns were similar for much of the 1 kb *hDHFR* upstream regulatory region; however, an increase in sensitivity to MNAse was observed at –470 bp upstream of the ATG and a number of other positions such as –320 and –120 in the active promoter (Figure 3A). Interestingly, this region of the calmodulin promoter is important in regulating transcription of the endogenous calmodulin gene (Crabb and Cowman, 1996). By using the same approach, we detected no differences in chromatin structure at a control locus (see Figure S1 in the Supplemental Data available with this article online). The data suggest activation of the inserted *hDHFR* gene is linked to increased accessibility of the transcriptional machinery to chromatin within the 5′ regulatory region of the promoter.

SIR2 histone deacetylase activity is required for heterochromatin silencing by promoting local alterations

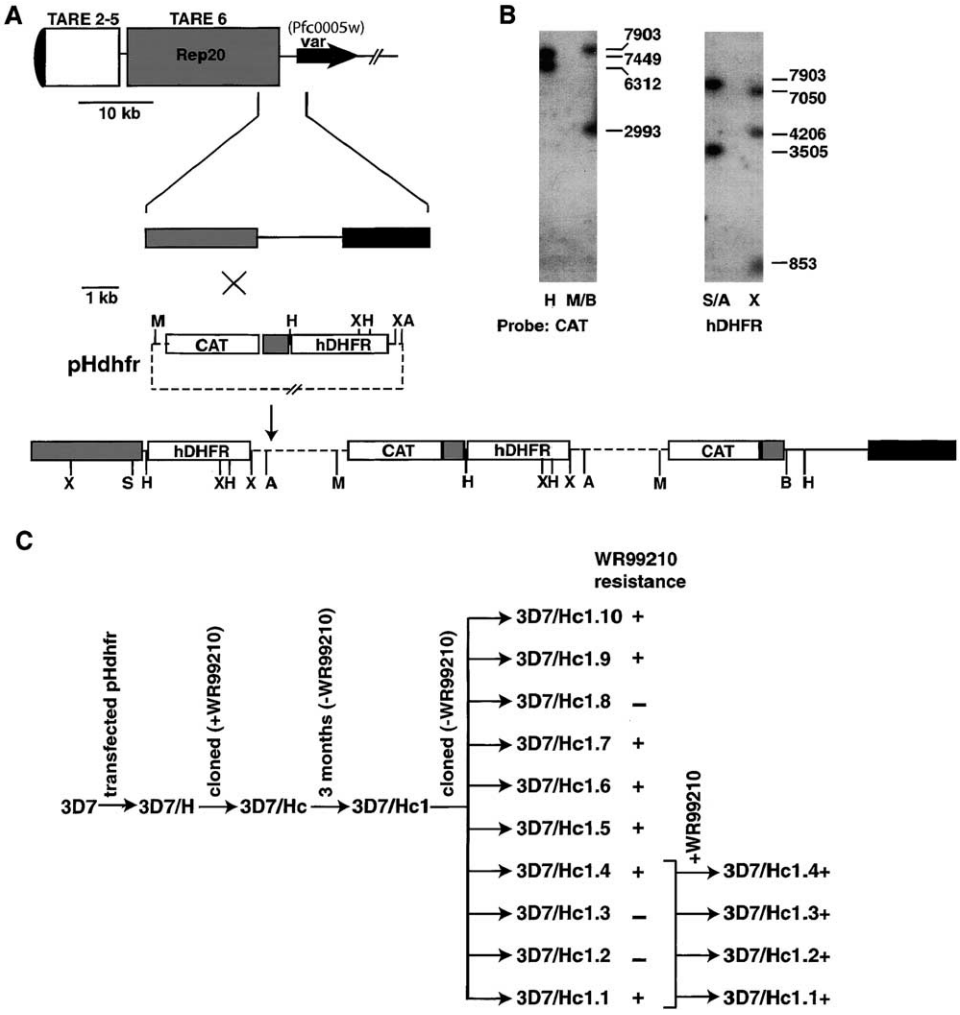

Figure 1. Structure of the *P. falciparum* Subtelomere for Chromosome 3 and Integration of *hDHFR*

(A) The conserved structure of the subtelomere of *P. falciparum* chromosomes is shown. Each chromosome consists of TAREs 1 to 6. The black region on the left end is the telomere. TARE6 corresponds to Rep20. The *var* on chromosome 3 (PFC0005w) is not transcribed in 3D7. The plasmid pHdhfr is shown and contains Rep20 for targeting. The restriction enzyme sites are: H, HindIII; M, MfeI; B, BsaAI; S, StuI; A, AatI; X, XbaI. Structure of the integration is shown (two copies of plasmid were inserted 3.3 kb upstream of the *var* ATG).

(B) Southern blots of gDNA from 3D7/H confirm integration of the plasmid into chromosome 3.

(C) Lineage of the 3D7 parasites transfected with pHdhfr. The *P. falciparum* clone 3D7 was transfected with pHdhfr (3D7/H). This transfected line was cloned with WR (3D7/Hc). This cloned line was grown for three months without drug (3D7/Hc1). 3D7/Hc1 was cloned without drug and the 3D7/Hc1.1–10 clones obtained. These were tested for growth in 10 nM WR and sensitivity after 3 days, indicated by the +/– symbols. The parasite clones 3D7/Hc1.1–4 were selected on WR to give 3D7/Hc1.1–4^{+}.

in chromatin compaction (reviewed in Lustig, 1998). To determine if SIR2-mediated gene silencing is involved in *var* gene regulation, we constructed parasites in which *PfSIR2* was disrupted (3D7Δsir2) (Figure S2A). Disruption of the gene was confirmed by Southern blots and restriction mapping (Figure S2B). Additionally, Northern analysis showed that the *PfSIR2* transcript was not detectable in the transfected parasites (3D7Δsir2; Figure S2C). Microarray experiments comparing global transcription of wild-type versus Δsir2 ring stage parasites showed a significant increase in transcripts for a subset of *var* and *rifin* genes, while most of the genes remained unchanged (Figure 3B; Table S1). *Var* genes are controlled by three main promoter types, defined as *upsA*, *B*, and *C* (Gardner et al., 2002). The genes regulated by *upsA* and *upsB* promoters are generally located in the subtelomeric region, although some are present in central regions; *upsC*-type *var* genes are exclusively found in chromosome-central clusters. The *var* genes that showed the highest increases in transcription were those controlled by *upsA* promoters. Furthermore, one subtelomeric *var* gene flanked by a distinct promoter (*upsE*) was also highly upregulated. Interestingly, transcription of a significant number of *rifin* genes was also increased in the absence of PfSir2, and these were generally in close proximity to the *var* genes controlled by *upsA* and *upsE* promoters.

To validate the altered *var* gene transcription pattern in Δsir2 parasites, we used Northern blots of ring stage RNA with probes for *var* genes *var2CSA* (PFL0030c)

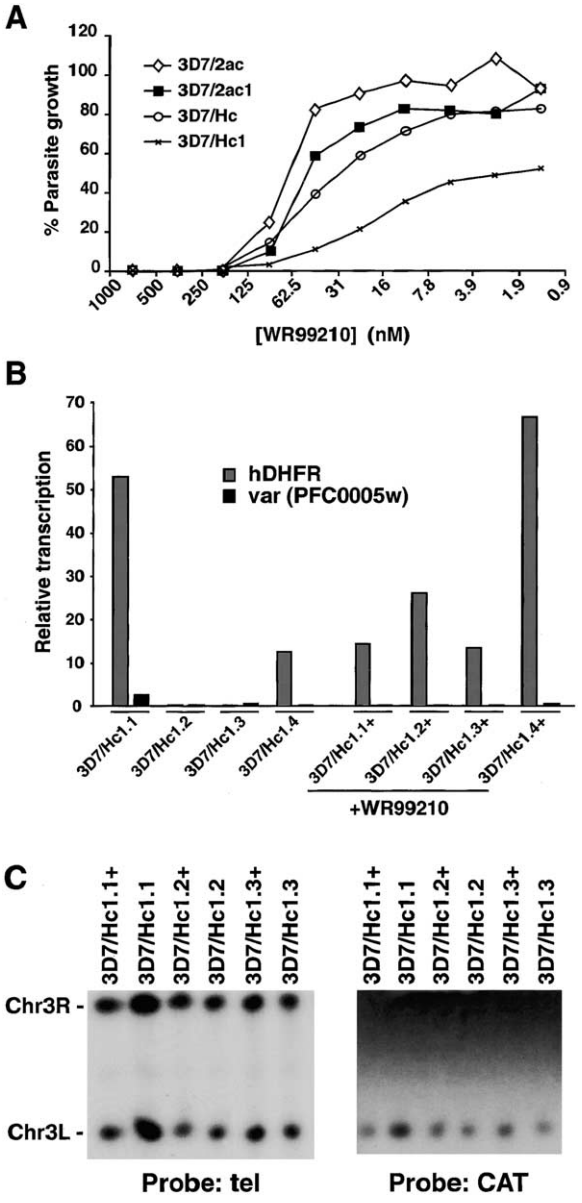

Figure 2. Characterization of pHdhfr Transfectants

(A) Sensitivity of 3D7 transfectants to WR. Drug curves were derived for 3D7/Hc and 3D7/Hc1 (derived from 3D7/Hc by 3 months of culture minus WR). These curves were compared to a control transfected line 3D7/2ac, and its derivative 3D7/2ac1 that was similarly obtained by 3 months growth minus WR. This transfectant possesses *hDHFR* targeted to the *PfRh2a* gene (Duraisingh et al., 2003).

(B) Transcription of *hDHFR* and the adjacent *var* (PFC0005w) in 3D7/Hc1.1, 3D7/Hc1.2, 3D7/Hc1.3 and 3D7/Hc1.4 compared to the same lines selected on 10 nM WR. The level of transcription was assessed relative to *KAHRP*.

(C) Chromosomes were separated by PFGE, and chromosome 3 was purified for digestion. Chromosome 3 digests were probed with telomere (tel) or chloramphenicol acetyl transferase (CAT) for 3D7/Hc1.1^{+}, 3D7/Hc1.1, 3D7/Hc1.2^{+}, 3D7/Hc1.2, 3D7/Hc1.3^{+}, and 3D7/Hc1.3. Bands corresponding to the right and left ends of chromosome 3 are indicated (Chr3R and Chr3L, respectively).

(*upsE*) and *PF13_0003* (*upsA*), both of which showed increased transcriptional levels using microarrays. To detect global *var* transcripts, we used a conserved exon 2 probe. The wild-type parasites show predominantly two major transcripts whereas Δsir2 parasites had a more complex pattern consistent with increased transcription of a larger number of *var* genes (Figure 3C). A large *var2CSA* transcript was detected in 3D7Δsir2, in contrast to wild-type parasites where none were observed. The *var* gene *PF13_0003* could be detected in both wild-type and Δsir2 parasites; however, in the latter it was present at higher levels (Figure 3C). It was possible that disruption of PfSir2 expression had caused a greatly increased *var* gene switch rate rather than general loss of silencing and as a result transcriptional activation of a large subset of this gene family within the population. To distinguish between these possibilities, we selected Δsir2 and wild-type parasites for adherence to CSA. The Δsir2 and Δsir2/sel (selected for adherence to CSA) parasites expressed the same complex pattern of transcripts consistent with general derepression of the *var* gene family including gene(s) encoding PfEMP1 proteins that bind the CSA ligand. In contrast, wild-type parasites showed a typical transcription pattern of two smaller *var* transcripts. When selected for adherence to CSA, the resulting parasites (3D7sel) showed a major transcript of >10 kb. This transcript was the same size as that observed for the *var2csa* gene in 3C, a parasite line derived from 3D7 by selection for CSA adherence (Duffy et al., 2005). Therefore, the complex *var* gene transcriptional pattern observed in the absence of PfSir2 results from a general derepression of this gene family in each parasite cell rather than an increase in gene switch rate within the population. These results are consistent with our microarray analyses and suggest that one tier of subtelomeric *var* gene regulation involves heterochromatin silencing for which PfSir2 is required.

Transcriptionally Active and Silenced Loci Occupy Different Compartments at the Nuclear Periphery

Evidence in other organisms has suggested not only that regulation of gene expression involves transcriptional elements but also that three-dimensional nuclear architecture exerts control by establishment of active and repressive subcompartments (Brown et al., 1999; Francastel et al., 2000; Gasser, 2001; Skok et al., 2001). This allows activation and repression of gene transcription by movement of specific chromosomal domains into different nuclear compartments (Gasser and Cockell, 2001). To test if *hDHFR* occupied a different spatial position within the *P. falciparum* nucleus when active compared to silenced, we employed FISH. To provide a marker of peripheral chromosome cluster activation, we generated a plasmid, pBsd/Rep20, that carried the blasticidin deaminase gene (*bsd)* conferring resistance to blasticidin-S, Rep20 to ensure association of the episome to a chromosome cluster at the nuclear periphery, and the luciferase (*lux*) gene as a probe (O'Donnell et al., 2002; Figure 4A). This plasmid was transfected into a WR-sensitive clone (3D7/Hc1.3) and selected on blasticidin-S until resistant parasites were obtained (3D7/Hc1.3/pB). The blasticidin-S-resistant line was subse-

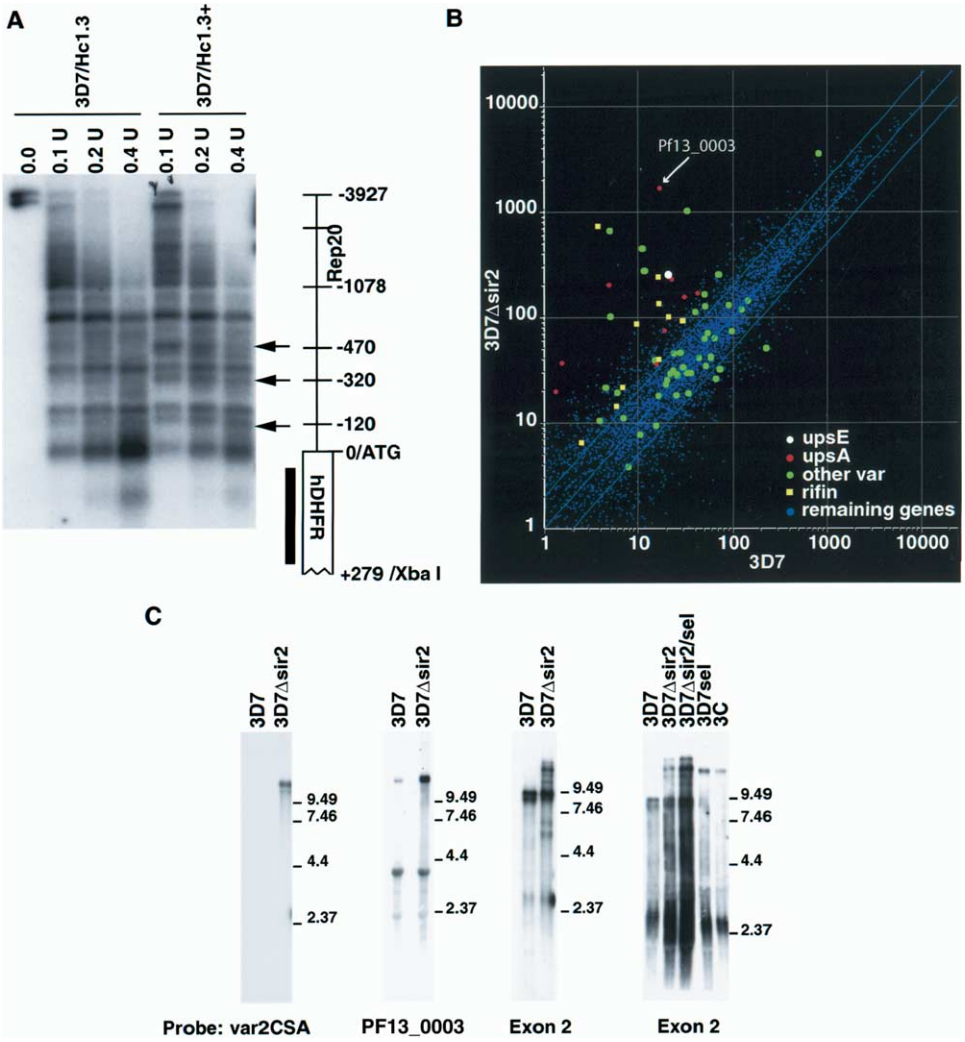

Figure 3. Chromatin Structure of *hDHFR* and Transcriptional Analysis of *P. falciparum* Lacking PfSir2 Function

(A) Chromatin structure analysis was performed by digestion of native chromatin with MNAse and indirect end labeling. MNAse-treated chromatin was digested with XbaI, and Southern blots hybridized with a 257 bp *hDHFR* probe. The MNAse cleavage sites enhanced in 3D7/Hc1.3^{+} are shown with arrows. Hybridization of the *hDHFR* probe to XbaI-digested intact chromatin (first lane) shows two bands (4201 and 7051 bp) corresponding to two tandemly integrated plasmids. The distance from the ATG start codon of the *hDHFR* gene is shown.

(B) Comparison of global transcription in ring stages of 3D7 and 3D7Δsir2. *Var* genes are shown as white (*upsE*), red (*upsA*), and green dots (other *var* genes). *Rifin* genes are shown as yellow dots, while the remaining genes are shown in blue. x and y axes are logarithmic and correspond to relative signal of hybridization to each gene with spots above the diagonal, suggesting increased transcription while below decreased transcription.

(C) Northern blot analysis to detect *var* gene transcripts in 3D7 and 3D7Δsir2. First panel: *var2CSA* probe detecting transcript from this subtelomeric gene (controlled by the *upsE* promoter). Second panel: *PF13_0003* probe detecting transcript from this subtelomeric gene (controlled by an *upsA* promoter). Third panel: exon 2 probe hybridizes to all *var* transcripts and detects global transcription of this gene family. The first and third panels are the same RNA filter successively hybridized after stripping the probe. Panel 4: exon 2 hybridized to RNA from 3D7, 3D7Δsir2, 3D7Δsir2 after selection for adherence to CSA (3D7Δsir2/sel), 3D7 after selection for adherence to CSA (3D7sel) (this study), and 3C a parasite line previously selected from 3D7 for adherence to CSA. Equal amounts of RNA were loaded in each track. Size of the RNA markers is shown in kb on the right of each panel.

quently selected on WR while also maintained on blasticidin in order to ensure activation of *hDHFR* transcription (3D7/Hc1.3^{+}/pB).

To confirm the transfected parasites contained the episomal pBsd/Rep20, we used Southern analysis on DNA from the WR-sensitive/blasticidin-S-resistant (3D7/Hc1.3/pB) and WR-resistant/blasticidin-S-resistant (3D7/Hc1.3^{+}/pB) parasite lines (Figure 4B). Hybridization with pGem detected the integrated *hDHFR* in chromosome 3 in all transfectants. As expected, the *lux* probe hybridized to the plasmid pBsd/Rep20 in the blasticidin-S-resistant parasites. To show that this plasmid was maintained episomally, we used PFGE of chromosomes from wild-type and transfected lines. The *bsd* probe hybridized only to the blasticidin-S-resistant parasites 3D7/Hc1.3/pB and 3D7Hc1.3^{+}/pB, and the pattern ob-

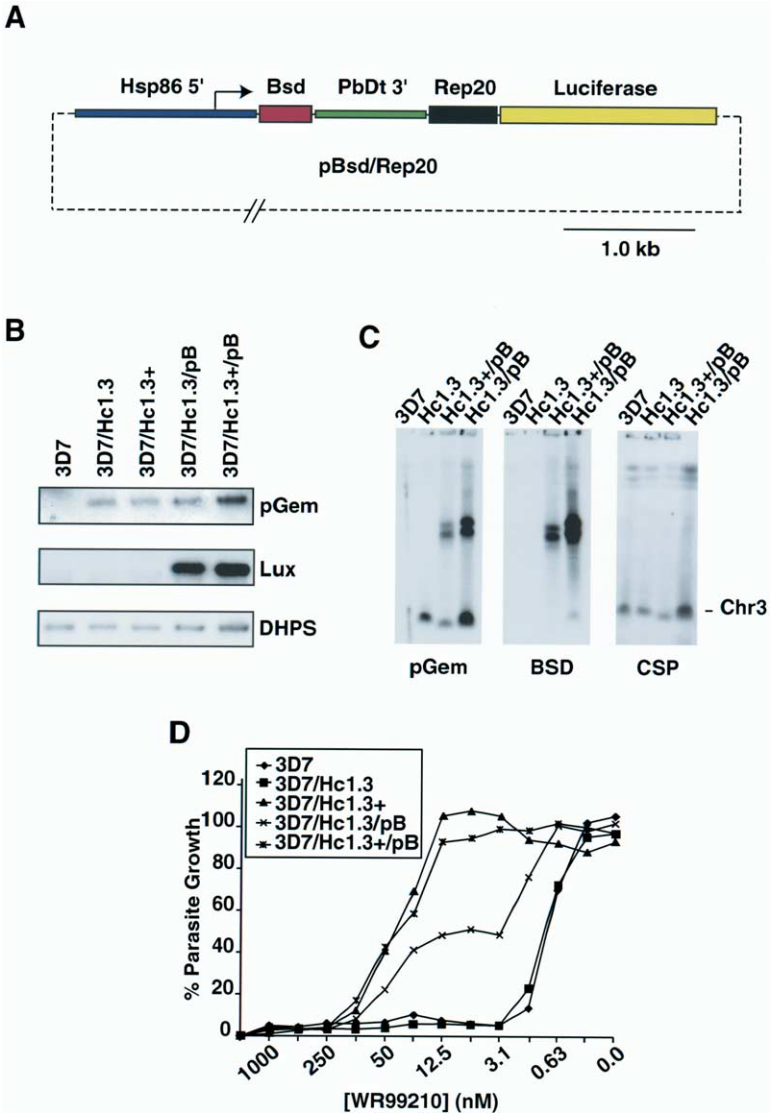

Figure 4. Characterization of Parasites Transfected with pBsd/Rep20

(A) The pBsd/Rep20 structure is shown. The *bsd* gene is controlled by *hsp86* promoter and flanked by the *P. berghei dhfr* terminator region (PbDt 3′). We included the luciferase gene as a hybridization target.

(B) Southern blots of genomic DNA from 3D7, 3D7/Hc1.3, 3D7/Hc1.3^{+}, 3D7/Hc1.3/pB, 3D7/Hc1.3^{+}/pB. Top panel: the pGem probe was used to probe EcoRI/EcoRV-digested genomic DNA. Center panel: the same genomic DNA was probed with luciferase probe (Lux) to detect the plasmid pBsd/Rep20 in 3D7/Hc1.3^{+}/pB and 3D7/Hc1.3/pB, but not in the other lines. Bottom panel: the dihydropteroate synthase (DHPS) probe was a loading control and present in all parasites.

(C) PFGE showing that the Bsd plasmid is episomal. Chromosomes from 3D7, 3D7/Hc1.3 (Hc1.3), 3D7/Hc1.3^{+}/pB, and 3D7/Hc1.3/pB were separated by PFGE and probed with pGem (first panel), blasticidin deaminase gene (BSD, second panel) or the circumsporozoite gene (CSP, third panel).

(D) WR sensitivity curves for 3D7, 3D7/Hc1.3, 3D7/Hc1.3^{+}, 3D7/Hc1.3/pB, and 3D7/Hc1.3^{+}/pB parasite lines.

served was that expected for an episome (Crabb and Cowman, 1996; O'Donnell et al., 2002). However, there was some hybridization of the *bsd* probe to chromosome 3 for 3D7/Hc1.3/pB parasites, suggesting partial integration had occurred by homologous recombination. Despite this, it appeared that the majority was migrating as an episomal plasmid. The pGem probe hybridized to chromosome 3 in all transfectants (corresponding to the integrated pHdhfr plasmid), as well as to the episomal plasmids in the relevant lines (Figure 4C). In contrast, the CSP probe hybridized to the corresponding gene present in all parasite lines on chromosome 3.

As expected, and identical to parental 3D7, 3D7/Hc1.3 parasites were WR sensitive, consistent with a silenced *hDHFR* (Figure 4D). In comparison, 3D7/Hc1.3^{+} was resistant to WR challenge, consistent with expression of *hDHFR* in these parasites (Figure 2B). Consequently, the 3D7/Hc1.3/pB parasites were expected to have a silenced *hDHFR* and to be highly sensitive to WR. However, we obtained a biphasic curve, suggesting a mixed population with approximately 50% resistant to WR, whereas the rest of the parasites were sensitive to this drug. This indicated that *hDHFR* was transcribed in the 3D7/Hc1.3/pB population, and this was confirmed by RT-PCR (data not shown). Due to large regions of homology between the primary integrated plasmid and the episome, there appears to be some recombination at the chromosome 3 *hDHFR* (Figure 4C), leading to transcription of the locus and partial drug resistance in this subpopulation. Nevertheless, the majority of parasites in the population had an unintegrated plasmid, consistent with coexistence of a transcriptionally active pBsd/Rep20 plasmid with the silent integrated plasmid.

In *P. falciparum*, Rep20-containing episomes physically associate with peripherally located telomeric chromosome clusters, a process that promotes partitioning of plasmids during mitosis (O'Donnell et al., 2002). As parasites transfected with pBsd/Rep20 are grown on

blasticidin-S, the *bsd* gene must be expressed and the plasmid contained within a transcriptionally competent region. Therefore, this plasmid serves as a marker for transcriptionally active domains at the nuclear periphery. To determine if perinuclear transcription in *P. falciparum* was restricted to zones, we performed FISH on the *hDHFR*-active and *hDHFR*-silenced blasticidin-S-resistant lines, 3D7/Hc1.3^{+}/pB and 3D7/Hc1.3/pB, respectively.

We employed two color FISH using the *cat* gene (green) to specifically detect the subtelomeric *hDHFR* on chromosome 3, and the plasmid (red) to visualize both the *hDHFR* transgene locus and the episome (Figure 5A). Compared to the active chromosome 3 end in 3D7/Hc1.3^{+}/pB, the silenced end in 3D7/Hc1.3/pB was significantly more likely to be in a distinct and separate position at the nuclear periphery relative to the transcriptionally active episome (17% ± 1% to 51% ± 1%; p = 0.007; Figure 5B). Nuclear integrity was maintained as shown by measuring the diameter of nuclei in xy and z planes (Figure S3). If the presence of a parasite subpopulation in 3D7/Hc/pB with an activated *hDHFR* was taken into account (approximately 50%), where it would be expected that this gene would colocalize with the pBsd/Rep20 plasmid either because of integration or physical association with a transcriptionally active region, these results become highly significant. This suggests that in *P. falciparum*, silent and transcriptionally active subtelomeric loci occupy different perinuclear locations.

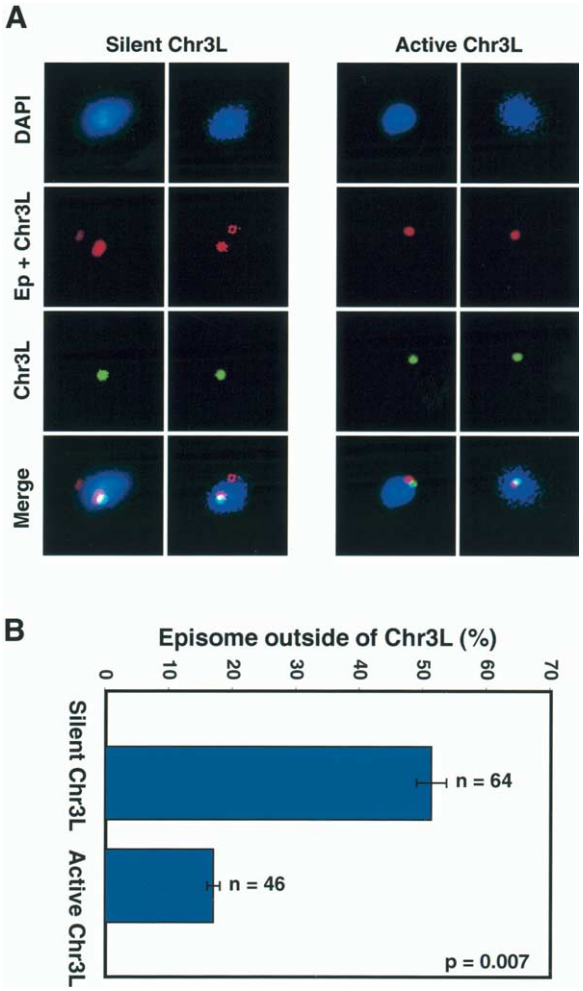

Figure 5. Nuclear Position of *hDHFR* on the End of Chromosome 3 Relative to the pBsd/Rep20 Episome

(A) FISH analysis of 3D7/Hc1.3/pB (silent Chr3L) compared to 3D7/Hc1.3^{+}/pB (active Chr3L). Epifluorescence images of nuclei stained with DAPI and hybridized with pGEM (red) to identify the position of both the pBsd/Rep20 episome and *hDHFR*, and CAT (green) to identify the position of integrated *hDHFR* on chromosome 3 only.

(B) Quantitation of nuclear localization of *hDHFR* in reference to the position of the pBsd/Rep20 plasmid. The % of times the plasmid pBsd/Rep20 (episome) does not colocalize with *hDHFR* on chromosome 3 (Chr3L). N refers to the average number of independent nuclei quantitated for each line. Images were randomized and counted blind by four individuals in two independent FISH experiments. Error bars are 95% confidence intervals (±1.96 × SEM). Standard deviation for Chr3L-active and Chr3L-silenced lines are 3.5 and 9.3, respectively. The p value was generated using a paired one-tailed t test.

Repositioning of the *var2CSA* Gene within the Nucleus Is Linked to Its Activation and Silencing

The parasite line CS2 was derived from E8B by selection for cytoadherence to chondroitin sulfate A (CSA), resulting in specific PfEMP1 expression encoded by the *var2CSA* gene located in the subtelomeric region of chromosome 12 (Salanti et al., 2003). However, in the unselected E8B parasite line, *var2CSA* was not transcribed at detectable levels. We used these parasite lines as a tool to determine if activation and silencing of *var* genes is linked to their position within the nucleus using the same strategy as above. The plasmid pHHM (pHHMC*/3R0.5) contained *hDHFR* and Rep20 for localization to "active" clusters at the nuclear periphery (O'Donnell et al., 2002). This plasmid was transfected into *P. falciparum* cloned lines E8B and CS2 to derive WR-resistant lines E8B-pHHM and CS2-pHHM, respectively. These parasites were analyzed to ensure retention of the parental cytoadherence properties to CSA, CD36, and ICAM-I. The CS2-pHHM parasites were as expected highly adherent to CSA, whereas E8B-pHHM showed no significant binding, and these properties are the same as observed for parental lines (Figure 6A). Similarly, the pattern of binding to both CD36 and ICAM-I was the same in parental lines and transfectants. Transcription of *var2CSA* in CS2-pHHM was confirmed by Northern blots (data not shown), showing that the transfected parasites predominantly transcribed this gene.

To determine if the active and silenced *var2CSA* gene occupied a different position within the nucleus, we used two color FISH analysis with specific probes that detected the episome (red) and *var2CSA* (green; Figure 6B). These results showed that in E8B-pHHM *var2CSA* was less likely to colocalize with the episome compared to CS2-pHHM (p = 0.003). E8B-pHHM displayed 33% ± 1% colocalization between the episome and *var2CSA*. FISH analysis of these lines with both Rep20 and TARE4 probes demonstrated there was an average of 4.5 telomere clusters per nuclei, and this predicts the

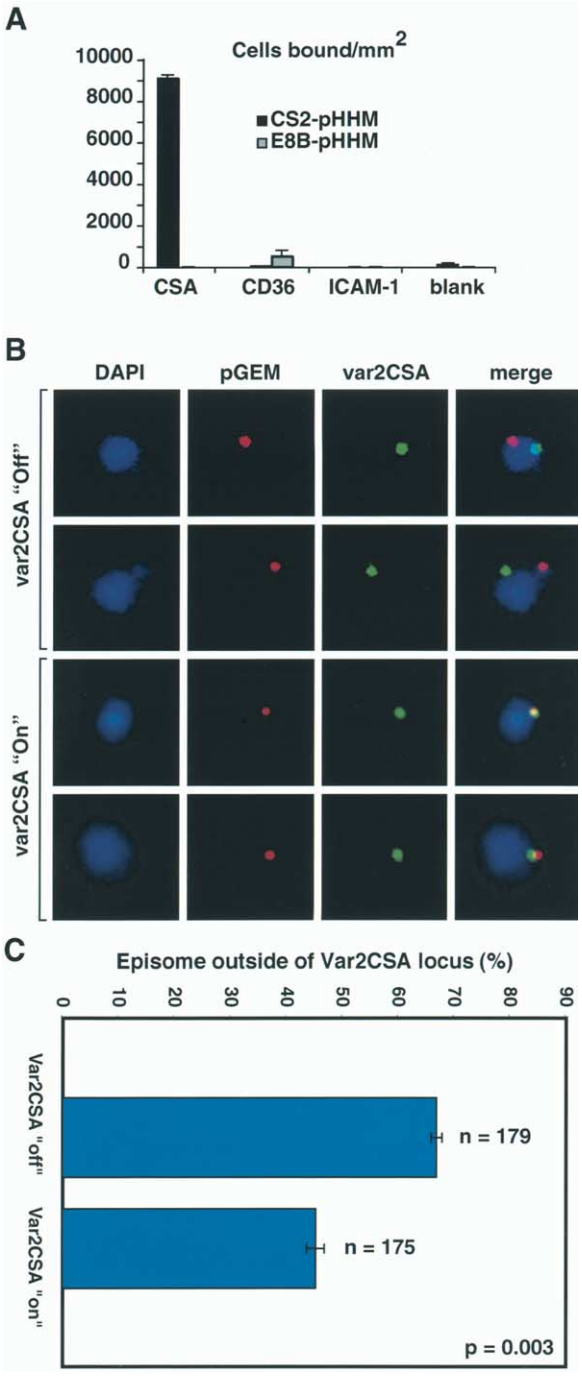

Figure 6. Cytoadherence and FISH Analysis of E8B and CS2 Transfected with an Episomal Plasmid

(A) Cytoadherence of E8B-pHHM and CS2-pHHM to CSA, CD36, and ICAM-I. *P. falciparum*-infected erythrocytes bound to the receptors were calculated and expressed as number of cells/mm^2. The pHHM (pHHMC*/3R0.5) plasmid was described previously (O'Donnell et al., 2002).

(B) Nuclear position of *var2CSA* on chromosome 12 relative to the pHHM episome in E8B-pHHM (*var2CSA* "Off") and CS2-pHHM (*var2CSA* "On"). Epifluorescence images of nuclei stained with DAPI and hybridized with pGEM (red) to identify the position of pHHM episomes and a specific *var2CSA* probe (green) to identify the gene on chromosome 12.

(C) Quantitation of nuclear localization of *var2CSA* in reference to the position of the pHHM episome. The % of times the episome does not colocalize with *var2CSA*. N refers to the average number of independent nuclei quantitated for each line. Error bars are 95% confidence intervals (±1.96 × SEM). The standard deviation for the E8B-pHHM and CS2-pHHM lines are 6.7 and 10.6, respectively. The p value was generated using a paired one-tailed t test.

random chance of an episome overlying any cluster was 22%, a figure close to that observed for colocalization of pGem and *var2CSA* in the unselected E8B-pHHM line. In contrast, CS2-pHHM shows a significantly higher colocalization for these probes (55% ± 1%; Figure 6C). Although one would expect this value to be higher, our findings are consistent with (1) the high switch rate measured for *var* genes (2% per generation), (2) the likelihood that not all of the CS2-pHHM parasites express the *var2CSA* gene due to the process of selection on chondroitin sulfate A and subsequent growth for a number of generations, and (3) expression of *var* genes other than *var2CSA* may confer a CSA binding phenotype as well. Nuclear integrity was again monitored for these experiments and confirmed as described (Figure S3). These results indicate that the silenced *var2CSA* occupies a distinct perinuclear position and activation involves repositioning to a new site.

Discussion

In this work, we show that genes located in the subtelomeric region of a *P. falciparum* chromosome can be reversibly silenced by epigenetic mechanisms and that active and silenced states occupy different nuclear positions. This indicates that spatial organization of genes within the nucleus and locus repositioning is linked to heritable virulence gene silencing in *P. falciparum*. We furthermore demonstrate, for the first time in *P. falciparum*, that transcriptional activation of subtelomeric genes involves changes in local chromatin structure and that SIR2-mediated silencing regulates *var* and *rifin* gene expression in these chromosomal regions.

We inserted a drug resistance marker into Rep20 of a *P. falciparum* chromosome adjacent to a *var* gene to specifically address transcriptional regulation of subtelomeric genes. This strategy enabled enforced expression of *hDHFR* and determination of alterations in the transcriptional state of this transgene when drug selection was removed. The gene silencing observed for *hDHFR* is a consequence of epigenetic regulation and independent of the promoter driving its expression. Also, silencing entails the assembly of repressive chromatin domains that are less accessible to probes such as MNAse (Telford and Stewart, 1989) and sites in the calmodulin promoter that are sensitive to MNAse digestion in the active state are protected against digestion when the promoter is silenced. Furthermore, the comparison of the degree of chromatin digestion at *hDHFR* and the control locus suggests that overall chromatin packaging at the silenced *hDHFR* transgene locus was more compact than at the active promoter. The *hDHFR* gene was stably silenced over generations through many mitotic divisions and shows epigenetic inheritance

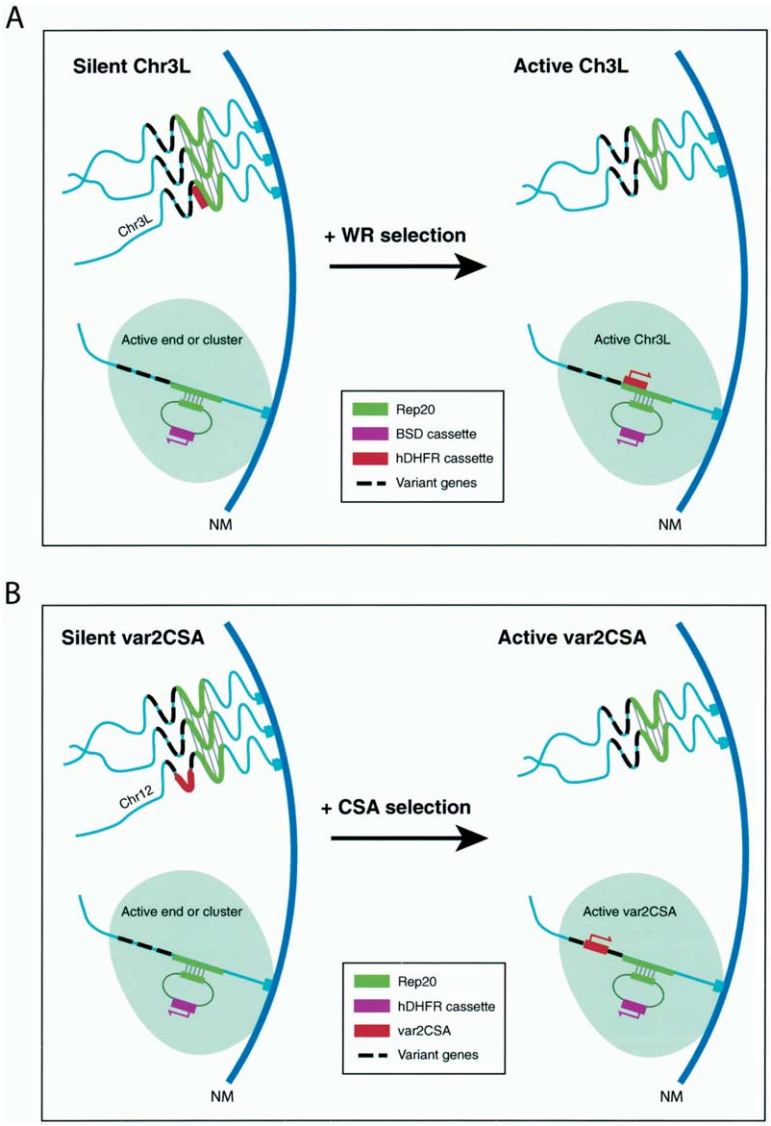

Figure 7. A Model of Locus Repositioning and Gene Silencing for the Subtelomeric Region of *P. falciparum* Chromosomes

(A) Left: in nuclei with an inactive *hDHFR*, Chr3L is separated from the transcriptionally active perinuclear episome in telomeric clusters tethered to the nuclear membrane (NM) (Freitas-Junior et al., 2000). Cluster formation, mediated by the TAREs, appears to involve Rep20 crosslinking (gray lines; O'Donnell et al., 2002). Given that in native chromatin the promoter in front of the inactive *hDHFR* is less accessible to micrococcal nuclease, it is likely that the silenced Chr3L end is packaged into heterochromatin (represented by wavy chromosomes). Here, the transcriptionally active BSD plasmid tethers to Rep20 of another chromosome end which is in a transcriptionally active peripheral subcompartment. This end is either on its own or itself in a cluster. Right: following activation of *hDHFR* transcription by WR selection, both the transcriptionally active Chr3L and the episomal plasmid colocalize. Hence, Chr3L appears to have relocated to the transcriptionally competent subcompartment occupied by the episomal plasmid. Furthermore, increased accessibility to endonuclease suggests the *hDHFR* promoter is in a more open conformation. It is important to note that the neighboring *var* is not activated in this instance, consistent with further layers of transcriptional regulation (e.g., promoter-intron cooperative silencing; Deitsch et al., 2001; Voss et al., 2003).

(B) Left: the clusters of chromosome ends are tethered to the nuclear periphery and associate via Rep20-interacting proteins and are located within a "silent" region of the perinucleus. The pHHM plasmid requires expression of hDHFR and must associate with an "active" cluster via Rep20. Activation of *var2CSA* by selection of E8B for adherence to CSA to derive CS2 results in relocalization of the end of chromosome 12 from a silenced cluster into a transcriptionally active region where the pHHM plasmid also resides due to the requirement for an active *hDHFR*.

although there must be a low rate of gene activation within the population to allow selection of drug-resistant parasites (Lustig, 1998). The expression properties of the *hDHFR* transgene are analogous to the silencing and activation of *var* genes and assist in understanding some of the important processes that regulate this virulence gene family.

In yeast, gene silencing occurs by formation of specialized chromatin structures involving a complex combination of *cis*-acting sites, *trans*-acting factors, and histones (Gasser and Cockell, 2001; Grewal and Moazed, 2003; Moazed, 2001). The SIR complex is important for silencing, and subsequently many other factors have been shown to be involved. The sequence of the *P. falciparum* genome is now available and most of the proteins involved in epigenetic silencing appear to have homologs in this protozoan parasite (Aravind et al., 2003; Gardner et al., 2002). Indeed, the notable paucity of specific transcription factors suggests epigenetic mechanisms of regulation may a play a prominent role in gene control in this organism (Aravind et al., 2003). We have demonstrated that disruption of *PfSIR2* leads to increased transcription of members of the *var* and *rifin* gene families and that PfSir2 is involved in regulation of virulence genes located in the subtelomeric region. This is consistent with the presence of these proteins at the ends of *P. falciparum* chromosomes as shown in the accompanying article (Freitas-Junior et al., 2005, this issue of *Cell*). Importantly, and consistent with our findings, PfSir2 was associated with the silenced *var2csa* gene but absent from its promoter in the active gene.

Activation of subtelomeric *var* and *rifin* genes as a result of PfSir2 loss of function suggests that, for at least a subset of these gene families, silencing is mediated by epigenetic mechanisms conserved in eukaryotes. Presumably, this is initiated by the histone deacetylase activity of PfSir2 followed by recruitment of additional silencing factors and the establishment of a heterochromatin-like environment preventing interac-

tions between the promoter and DNA binding factors. This hypothesis is consistent with the observed decreased accessibility of MNAse at the silenced *hDHFR* promoter. Activation of predominantly subtelomeric *var* genes with *upsA* and the unique *upsE* promoters suggests that the other members of this gene family are regulated by distinct mechanisms or have additional layers of regulation in transcriptionally competent areas in the nucleus.

Both subtelomeric genes investigated in this study (*hDHFR* and *var2CSA*) have different positions within the nucleus depending on their state of activation, implicating a role for perinuclear repositioning in regulation of *var* genes. Subnuclear localization and locus repositioning is a feature of heritable gene silencing in a number of eukaryotic cell types (Brown et al., 1999; Feuerbach et al., 2002; Skok et al., 2001). *Trypanosoma brucei* variant surface antigens are encoded by the *vsg* multigene family, and monoallelic expression appears to be accomplished by a specific nuclear body that allows PolI transcription of the single VSG gene occupying this privileged site (Navarro and Gull, 2001). Entry of *var* genes into a privileged site that is competent for transcription would be consistent with the data presented; however, it is unknown if the nuclear periphery of *P. falciparum* has a distinct subcompartment, as in *T. brucei*, or if it comprises highly dynamic transcriptionally active domains. Until live imaging of telomeres is technically feasible in *P. falciparum*, we are not able to rule out the possibility of increased telomeric mobility in active ends leading to more frequent encounters between transcriptionally active areas.

Our data indicates that locus repositioning of subtelomeric *var* genes within the nucleus plays an important role in transcriptional regulation of this gene family and maintenance of monoallelic expression. Expression of a specific *var* gene would require its physical positioning within a nuclear domain competent for transcription and consequently, as there could be up to seven chromosome ends per cluster (Freitas-Junior et al., 2000), this would bring a number of *var* genes within this domain. Even if only one chromosome end occupied the "active zone," because multiple *var* genes are often present on the one end, it would be important to have additional tiers of regulation to ensure mutually exclusive expression, and this is consistent with maintenance of the silent state for the *var* gene adjacent to the active *hDHFR*. Current evidence suggests that *var* gene noncoding regions harbor important boundary elements that may insulate them from derepressed chromatin in an active compartment of the nucleus (Deitsch et al., 2001; Voss et al., 2003). This is supported by our data showing that transcriptional activation of the subtelomeric *hDHFR* transgene is associated with chromatin structural changes in the upstream calmodulin promoter. Such local chromatin modifications could involve acetylation of histones at discrete nucleosomal regions, as it has been shown to occur in the activation of the HV-1 LTR promoter (Lusic et al., 2003). It is also consistent with the demonstration that upon activation of a *var* gene in *P. falciparum*, the PfSir2 associated with the silent gene was removed from the promoter region and acetylation of histone 4 occurred (Freitas-Junior et al., 2005).

Inclusion of Rep20 into the transfection vector pBsd/Rep20 targets the episomal plasmid to chromosome end clusters at the nuclear periphery. Constant selection with blasticidin-S of the parasites transfected with this plasmid guarantees expression of the *bsd* gene, and hence nuclear localization of pBsd/Rep20 by FISH analysis identifies the active perinuclear chromosome end cluster. Based on this, we propose a model (Figures 7A and 7B) to summarize the current data where locus repositioning accompanies activation or silencing of subtelomerically located genes. In this model, a silent locus is localized in a peripheral nuclear region that is not conducive to transcription and has a condensed heterchromatic structure. The episomal plasmid necessarily localizes to a transcriptionally active area, and consequently its localization would be different from that of the silent gene. In contrast, a transcriptionally active gene colocalizes with the episomal plasmid to an active cluster at the nuclear periphery. Here, the chromatin structure in discrete regions of these chromosome ends would be more open and accessible to regulatory *trans*-acting factors, and we would predict an active *var* gene would be present in this cluster. However, the other *var* genes located in this "active" perinuclear environment would still be in a silenced state due to higher level tiers of regulation such as transcriptional boundary or insulator elements. It is not known if the active end is part of a cluster or moves away from a silenced cluster.

In summary, we have shown that subtelomeric genes in *P. falciparum* can be silenced in a stably heritable, yet variegated, fashion. Reactivation of the subtelomeric gene involves repositioning within the perinuclear region. The role of nuclear architecture in gene regulation appears to be evolutionarily conserved in many eukaryotes, and similarly it plays a key role in regulation of important virulence genes in malaria parasites.

Experimental Procedures

Plasmids, Drug Sensitivity, Transfection, and Analysis of *P. falciparum*

3D7 is a cloned line derived from NF54 obtained from David Walliker, University of Edinburgh, United Kingdom. Transfection of parasites was carried out as described previously (Duraisingh et al., 2003). E8B and CS2 are derived from an ITG2 line. Cytoadherence of E8B-pHMM and CS2-pHMM to CD36, chondroitin sulfate, and ICAM-I was performed as previously described (Duffy et al., 2002). The pHdhfr, pHHM (pHHMC*/3R0.5), and pH2a plasmids have been described previously (Duraisingh et al., 2003; O'Donnell et al., 2002). Sensitivity to WR was assessed using the standard microdilution technique.

Real-Time Polymerase Chain Reaction

RNA was purified by Trizol (Invitrogen) extraction followed by RNeasy minicolumn purification (Qiagen). cDNA was used to measure levels of *hDHFR* and *var* transcripts with Quantitect (Qiagen) on an ABI 9700. These measurements were standardized against the KAHRP cDNA to calculate relative expression ratios.

Chromatin Structure

Native chromatin was digested with MNAse in parasite cells made permeable with NP-40. Late stage parasites (3 ml packed parasitized red blood cells) were resuspended in 1 ml ice-cold permeability/chromatin digestion buffer (CDB) (20 mM Tris-HCl [pH 7.5], 15 mM KCl, 60 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 300 mM sucrose, 0.4% NP-40) and incubated on ice for 5 min. Five 200 μl aliquots

were equilibrated at 37°C for 2 min and then digested with MNAse (MBI Fermentas) for 3 min at 37°C by adding 5 μl of enzyme (0.16, 0.08, 0.04, 0.02, and 0 U/μl). Reactions were stopped with 40 μl stop buffer (100 mM EDTA, 4% SDS) and 66 μl 2.5 M NaCl. Samples were treated with 100 μg Proteinase K and 50 U RNase A for 2 to 4 hr at 50°C. Digested chromatin was extracted with phenol/chloroform and chloroform, precipitated with 2.5 vol 100% ethanol and resuspended in 30 μl TE buffer (10 mM Tris-HCl [pH8.0], 1 mM EDTA). For indirect end-labeling, 10 μl MNase-digested chromatin samples were digested to completion with restriction enzymes, resolved on a 1.3% agarose gel, blotted onto Hybond-XL (Amersham), and probed with *hDHFR* or *PfSIR2*.

FISH and Microarrays

FISH was performed essentially as previously described (Ersfeld et al., 1998) using either biotin-labeled (Promega pGEM T-Easy kit) or fluorescein-labeled (PCR products) probes prepared using Roche High-Prime Kits. The slides were quantitated using 60–100 fields containing 5–10 nuclei of each experiment. These were photographed using a Carl Zeiss Axioskop with a PCO SensiCam and Axiovision 2 software. Composite images were produced using Photoshop 6.0.1, and the images collated and randomly coded and scored independently by four to five individuals. To monitor nuclear integrity, 50 nuclei in each experiment were stained with DAPI and scanned using a Leica TCS.SP2 confocal microscope attached to a Leica DMIIRE 2 inverted microscope. As a control for nuclei that have compromised integrity, cells were lysed with 0.05% NP-40 for five minutes prior to fixation. Fourteen sequential 0.168 μm confocal sections of the nuclei were imaged, and nuclei were reconstructed three-dimensionally. The nuclear diameter in the xy and z plane of each nucleus was calculated from computer graphic representations of the fluorescent DAPI signals set at a uniform threshold intensity of 35.

For microarrays, total RNA was isolated from ring stages using the RNeasy Kit (Qiagen). Hybridization to a *P. falciparum* custom-designed Affymetrix chip and scanning was done as previously described to arrays representing the complete genome of *P. falciparum* (Le Roch et al., 2003). Data analysis and presentation were performed using GeneSpring (Silicongenetics).

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/121/1/13/DC1/.

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